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BIOSYNTHESIS AND TRANSFORMATION OF MICROSOMAL AND CYTOSOL ESTRADIOL RECEPTORS

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SUMMARY

Extracts of uterine microsomes contain a "basic" 3·5 S and an "acidic" 4·5 S estradiol receptor. The smaller "basic" molecule appears to be an early product of receptor biosynthesis. It dimerizes to a "basic" 4·5 S entity on heating. Both the artificial "basic" dimer and the extracted "acidic" 4·5 S molecule are reversibly dissociated by protonation into 3·5 S "basic" and 3·5 S "acidic" subunits respectively. The heat-accelerated formation of stable dimers requires monomer-bound estradiol. The dimers are also dissociated by 2 M urea. High-speed supernatant (cytosol) of uterine homogenates, prepared with either low ionic strength buffer, pH 7·5, or buffered 0·25 M sucrose, contains only "acidic" receptors sedimenting at 4 S and 5 S in sucrose gradients prepared with buffered 0·4 M KCl. The radioactivity sedimenting at 5 S is shifted to the 4 S position by protonation. The reverse shift by proton withdrawal is accelerated by heating. Similar to the estradiol requirement for the formation of stable microsomal 4·5 S dimers, stable cytosol 5 S-estrogen complexes are only derived from estradiol-charged 4 S molecules. In contrast to the microsomal 4·5 S dimer, the cytosol–estradiol 5 S complex is not affected by 2 M urea, which improves the resolution of the two cytosol peaks and avoids the formation of rapidly sedimenting aggregates on heating. The following sequence of receptor forms is indicated as the major *in vivo* pathway: "basic" microsomal 3·5 S → "acidic" microsomal 3·5 S → cytosol 4 S → cytosol 5 S.

Receptor synthesis in the uterus is not necessarily dependent on estradiol since it persists in the uteri of both ovariectomized and ovariectomized/hypophysectomized rats. The administration of estradiol to hormone-deprived animals results in a "depletion–replenishment" sequence of receptor levels, which is caused by irreversible utilization and resynthesis of receptors. Estradiol thus appears to enhance the rate of receptor synthesis.

INTRODUCTION

The intracellular proteins which bind estradiol with high affinity and specificity, the so-called receptors, are generally classified by their site of origin. Gorski and his co-workers [1] first described the presence of a "9 S" receptor in the high-speed supernatant of uterine homogenates prepared with low ionic strength buffer. In Jensen's group in Chicago, we extracted a "5 S" receptor from the crude nuclear fraction with neutral, 0·3 M KCl solutions [2]. Accordingly, the former protein was referred to as "cytosol" receptor, the latter as "nuclear" receptor. Their properties, interrelationship and involvement in the mechanism of action of estradiol have been the subject of extensive studies by numerous laboratories [3]. The large cytosol receptor contains one or several smaller estradiol-binding entities [4]; the nuclear receptor either totally or in part derives from the cytosol receptor after translocation into the nucleus [5].

We have extracted and characterized estradiol-binding proteins from the microsomal fraction of uteri [6]. It is the aim of this paper to demonstrate possible links between the various forms of structure-bound and soluble cytoplasmic receptors.

The experimental procedures used for the extraction of microsomal receptors have been published in detail [6, 7]. Important points are the presence of estradiol in the extractant and the use of low ionic strength phosphate rather than Tris buffer, which minimizes aggregation. In order to obtain sufficient quantities of microsomes, most experiments were done with pig uteri, some with calf uteri. The extracts were analysed by sucrose-density gradient centrifugation and by agargelelectrophoresis at low temperature [8]. The typical patterns of 2 individual extracts are shown in Fig. 1. The extracts were charcoal-treated prior to analysis to remove the excess of free estradiol. Density gradient analysis revealed 2 peaks, sedimenting at approximately 3·5 S and 4·5 S. The radioactivity sedimenting at 4·5 S corresponds to the estradiol complex which migrates towards the anode in agargelelectrophoresis at pH 8·2, while the 3·5 S peak corresponds to the protein-bound estradiol located at the cathodic side of the starting line. This peak is well separated from the peak of remaining free estradiol, which is shifted further towards the cathode by electroendosmosis.

By comparing the electrophoresis of a cytosol with that of a microsomal extract (Fig. 2), it becomes evident that the "basic" receptor is an unique feature of submicroscopic cytoplasmic structures, possibly an early product of receptor biosynthesis or secretion, which still lacks the attachment of some acidic group. Removal of this acidic group from "mature" receptors should then result in a decrease of their electrophoretic mobility and in the appearance of a single "cathodic" receptor peak.

Our first attempts to achieve this by incubating

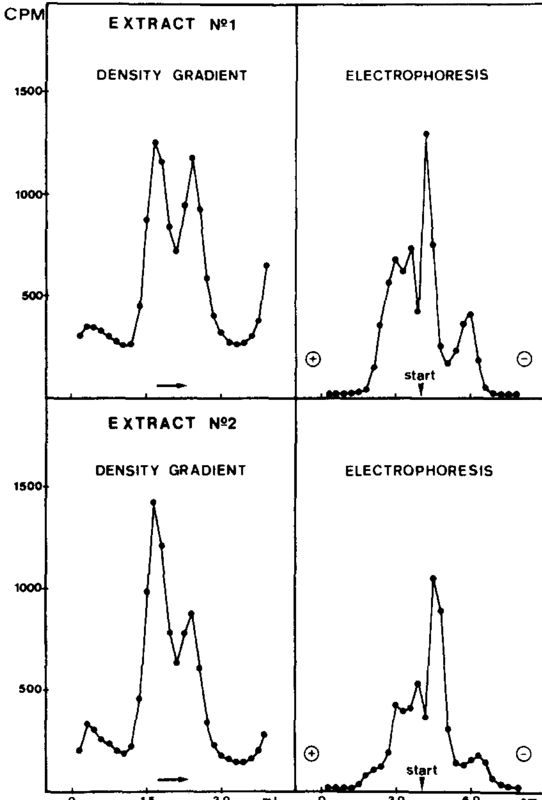

Fig. 1. Sedimentation velocities and electrophoretic mobilities of microsomal estradiol receptors. Pig uterine microsomes extracted with 0·01 M phosphate pH 7·5, 0·005 M NaN_3, 6×10^{-8} M [6,7^{3}H]-estradiol. Extract was charcoal-treated before analysis and 0·2 ml applied to density gradients 5–20% sucrose, 0·05 M phosphate pH 7·5, SW 56, 56,000 rev./min, 1°C, 19 h, 0·15 ml constant volume sampling from the top; 2 × 0·05 ml were applied to agargelelectrophoresis, 0·05 M Michaelis buffer pH 8·2, 120 mA constant current, 200–270 V, 20 min, 2–4°C in the gel, 3 mm slices.

microsomal extracts with neuraminidase for 30 min at 30°C had an unexpected result. Incubation with the enzyme left the electrophoretic pattern of extracts unchanged. But, on density gradient centrifugation of warmed extracts, both with and without enzyme, a marked translocation of radioactivity from the 3·5 S to the 4·5 S position was seen (Fig. 3). This is analogous to the observation made by Brecher *et al.*[9] with respect to the temperature-accelerated transition of a 4 S to a 5 S receptor in cytosol containing 0·4 M KCl. There are other similarities. For instance, neither the 3·5 S-estrone complex formed from microsomal extracts nor the 4 S-estrone complex from cytosol shift position after warming, the corresponding estriol complexes doing so only to a small extent. Considering these similarities and the minor differences in sedimentation coefficients, it might appear obvious that the 2 × 2 forms of the receptors are, in reality, only two. But the microsomal receptors exist at low ionic strength, the cytosol receptors do not and the small microsomal receptor has an electrophoretic mobility different from all other forms of receptor.

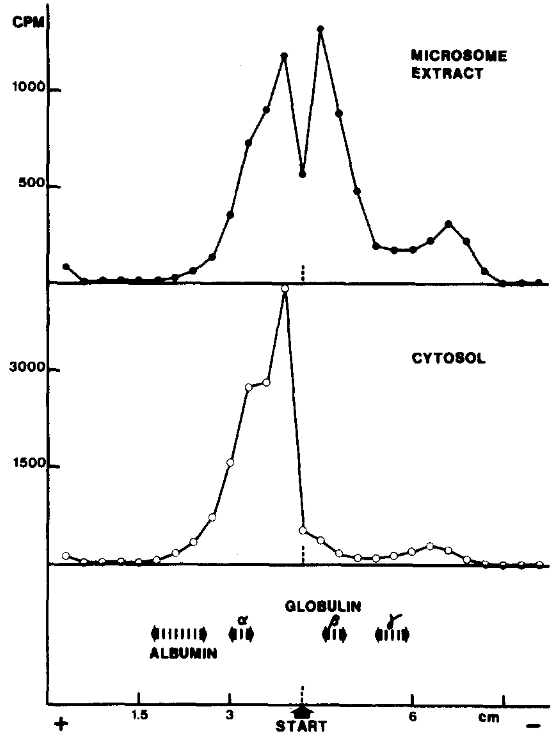

Fig. 2. Agargelelectrophoresis of pig uterine cytosol and microsomal extract. Cytosol = high speed supernatant of 1:1 homogenate with 0·25 M sucrose, 0·05 M phosphate, pH 7·5, 0·005 M NaN_3; incubation with 6×10^{-8} M [6,7^{3}H]-estradiol for 3 h at 2°C, charcoal treatment before analysis. For other conditions see legend to Fig. 1.

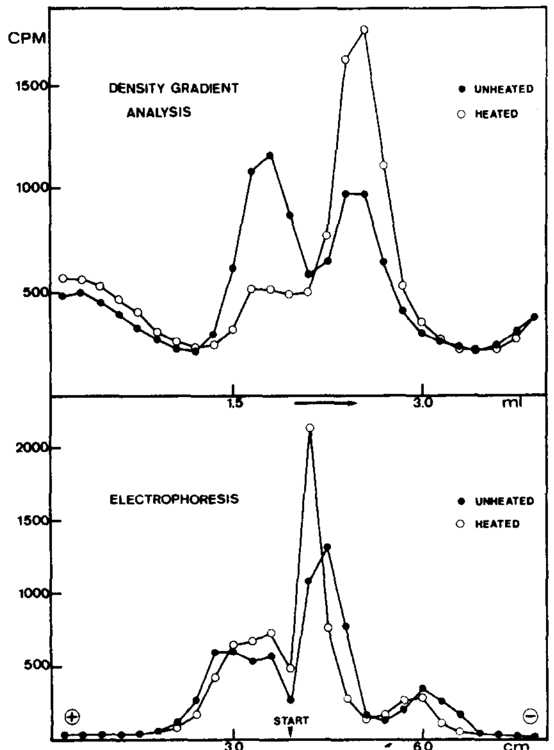

Fig. 3. Temperature accelerated 3·5→4·5 S transition of microsomal receptors. "Heated" was kept for 30 min at 30°C.

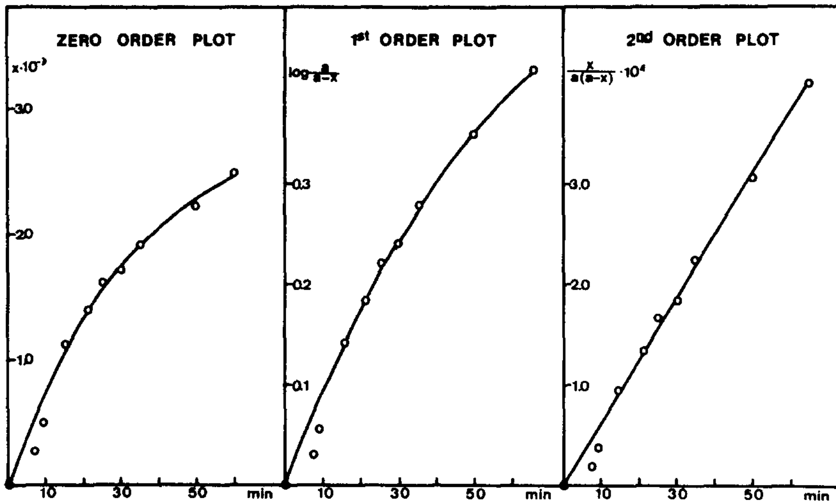

Fig. 4. Kinetics of temperature accelerated 3·5 → 4·5 S transition of microsomal receptors. Pig uterine microsomal extract incubated at 25°C.

The heat-accelerated transformation of the microsomal 3·5 S receptor to the 4·5 S form follows second order kinetics for dimerization, as Fig. 4 demonstrates. An indication of the possible types of bonding involved is given from the study of the stabilities of the two microsomal estradiol–receptor complexes at various pH values (Fig. 5). There is a sharp decline in the concentration of the 4·5 S molecule, accompanied by a rise in the concentration of the 3·5 S molecule, when the pH is lowered from 7·0 to 6·5. A sharp drop in the amount of the 4·5 S molecule occurs also between pH 10 and 11, here without a concomitant rise in the 3·5 S receptor concentration. The two pH ranges correspond to the pK's of histidyl and tyrosyl residues. Hydrogen-bonding between these two groups is therefore a reasonable assumption, which is supported by the reversible transformation by proton addition and withdrawal (illustrated in Fig. 6), and by the instability of the 4·5 S microsomal receptor in 2 M urea which totally transforms it to the 3·5 S molecule.

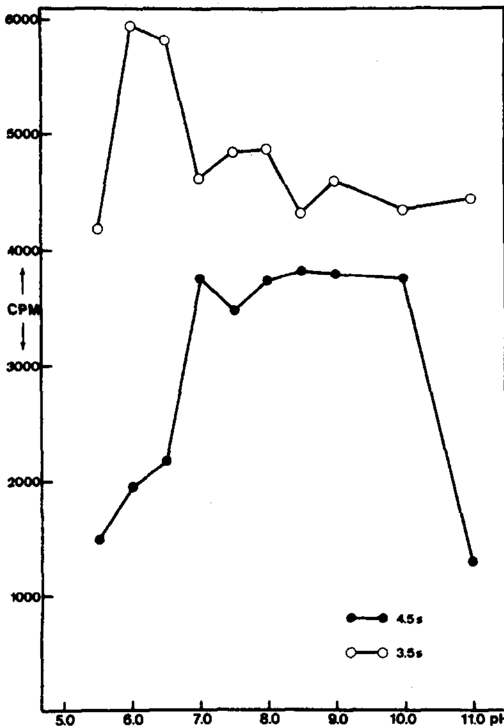

Fig. 5. Stability of 3·5 S and 4·5 S microsomal receptor to pH. Extract prepared with 0·01 M citrate/phosphate/borate buffer pH 7·5; density gradient centrifugation in 5–20% sucrose gradients containing 0·05 M citrate/phosphate/borate buffers pH 5·5–11.

Before having a closer look at the receptors in the high-speed supernatants, the cytosol receptors, it should be re-emphasized that all forms of cytoplasmic receptors, the structure-bound ones and the soluble ones, bind estradiol with the same affinity and therefore should contain identical estradiol-binding sites.

The well-known sedimentation pattern of cytosol receptors at low ionic strength (Fig. 7—the electrophoretic analysis in the lower half of this figure again shows the lack of a "basic" receptor) is changed after the addition of salt (Fig. 8) and reveals two peaks sedimenting at about 4 and 5 S. Both forms—in contrast to the microsomal receptors—are stable in the presence of 2 M urea which also does not interfere with the already mentioned 4 → 5 S transition on warming. The kinetics of this transition could not be as satisfactorily established as those for the microsomal receptors, as Fig. 9 shows. It might very well follow second order for dimerization, if one considers that the last values are prone to experimental error, because of the poor separation of the remaining small amounts of the 4 S entity.

That similar bonds are involved in the association process is suggested by a pH dependent transition between the two cytosol receptors (Fig. 10), taking place in the same pH range as seen with the microsomal receptors. This phenomenon has already been

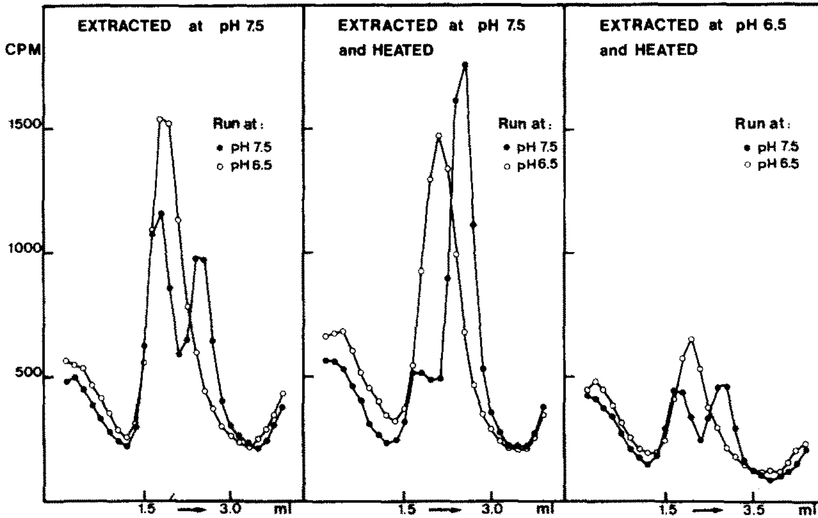

Fig. 6. Interconversion of microsomal estradiol-receptor-complexes by proton addition and withdrawal. Extracts were prepared at the pH values indicated on the graphs and then run at pH 6·5 (○——○) and 7·5 (●——●), respectively.

described by Notides and Nielsen in their study of the cytoplasmic receptors [10].

The known properties of the microsomal and the cytosol receptors are summarized in Table 1. The sedimentation coefficients shown are denominators rather than accurate values. Notides and Nielsen[10] measured 4·2 S and 5·5 S, respectively, for the cytosol receptors in sucrose gradients containing 0·4 M KCl. The sedimentation velocity of the microsomal receptors is identical in the absence and in the presence of 0·4 M KCl in density-corrected gradients. Estima-

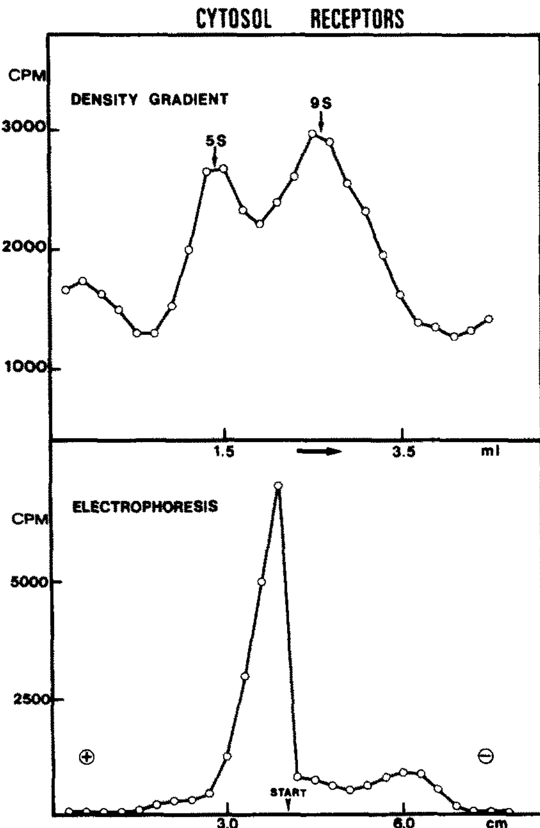

Fig. 7. Sedimentation velocities and electrophoretic mobility of pig uterus cytosol receptors at low ionic strength.

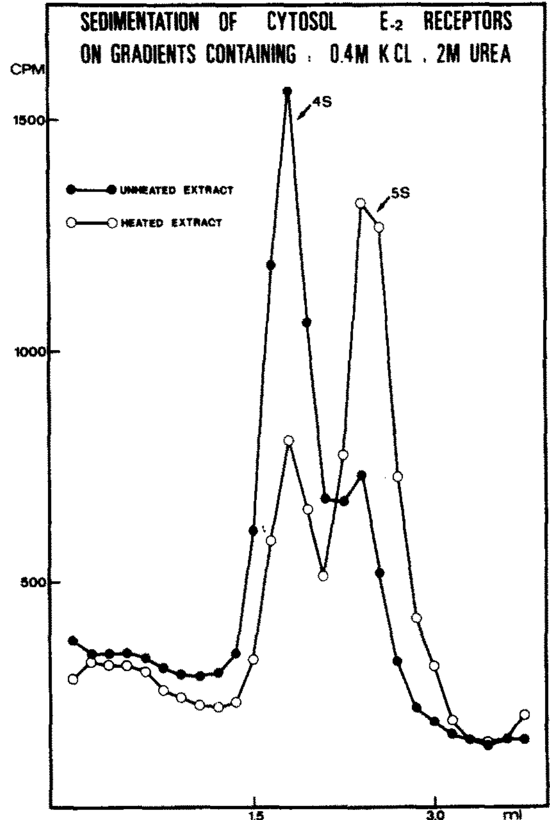

Fig. 8. Sedimentation velocities of pig uterus cytosol receptors at high ionic strength, temperature accelerated 4→5 S transition. Extracts were made up to 2 M urea, 0·4 M KCl, "heated" was kept for 70 min at 25°C; 5–20% sucrose gradients contained 2 M urea, 0·4 M KCl, 0·01 M phosphate pH 7·5; 25 h, SW 56, 56,000 rev./min, 1°C.

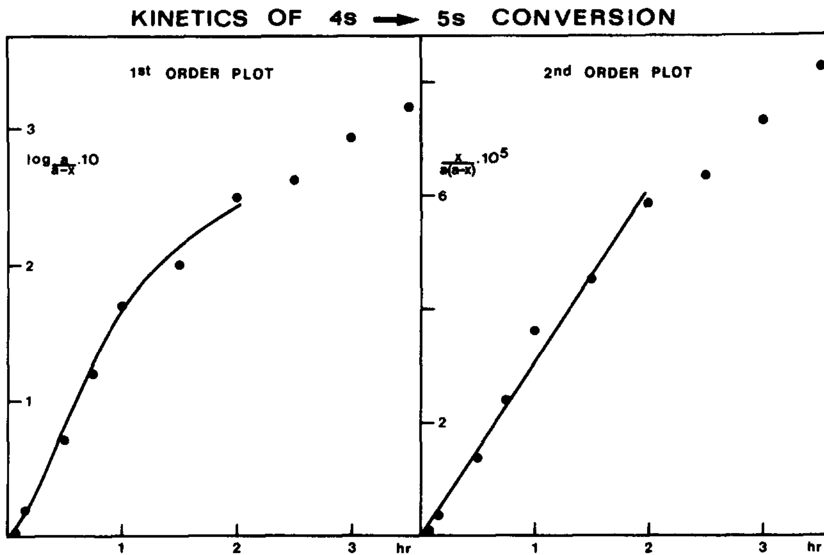

Fig. 9. Kinetics of temperature accelerated 4→5 S transition of cytosol receptors. Calf uterus cytosol, 0·4 M KCl, incubated at 10°C.

tion of the molecular weights by gel filtration—the figures for the cytosol receptors again are taken from Notides and Nielsen—stresses the close resemblance in size of the 4·5 S microsomal and the 4 S cytosol receptor. Doubling of the molecular weights of the small units falls short by 13,000 in the case of the microsomal receptors and by 19,000 in the case of the cytosol receptors. With the exception of the 3·5 S microsomal receptor, all other forms display the same electrophoretic mobility. Estrogen binding specificities of the two small receptor forms and the requirement of estradiol for the temperature accelerated transition to their large counterparts are identical. A second order dimerization kinetics of this transition is better established for the microsomal receptors than for the cytosol receptors. The participation of similar bonds in the transition can be concluded from the pH effects. Accessibility to those bonds must be different since urea only dissociates the microsomal 4·5 S receptor but leaves the cytosol 5 S receptor unharmed and even allows for the 4 S→5 S transition.

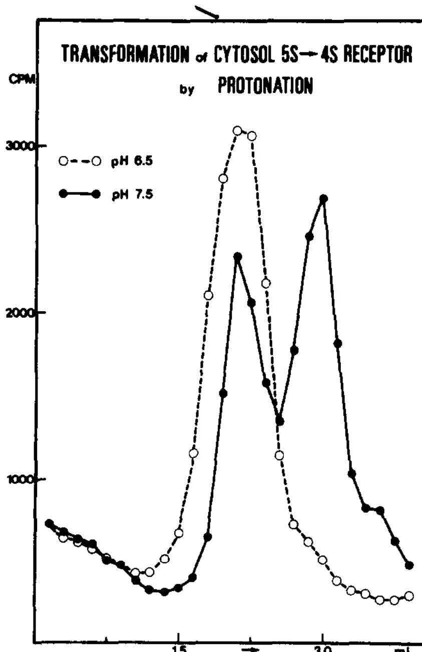

Fig. 10. Transformation of cytosol 5→4 S receptor by protonation. Calf uterus cytosol adjusted to and run at pH values indicated.

Although it seems improbable that all these *in vitro* observed properties and reactions could be of no physiological significance, positive proof can only be obtained from *in vivo* experiments. For this, we chose the receptor depletion–replenishment response in ovariectomized animals after a single injection of estradiol. An important prerequisite for the interpretation of results is the answer to the question of whether depletion means using up and replenishment stands for receptor resynthesis. In estradiol-primed, ovariectomized rats, the concentration of free cytoplasmic receptor measured as estradiol–receptor complex after incubation of the cytosol at 2°C with an excess of labelled hormone, falls to a minimum at about 5 h after the i.p. injection of 1 μg of cold estradiol and then rises to or above the preinjection level (Fig. 11). Treatment of aliquots of the cytosols with charcoal to remove free and unspecifically bound cold estradiol and subsequent incubation with an excess of labelled estradiol for 30 min at 30°C essentially yields the same receptor concentrations. (Under these conditions, an exchange of hot and cold estradiol at the receptor binding site can be achieved *in vitro* [11].) Therefore, *in vivo*, estradiol-tagged receptor must be removed from the cytoplasm almost instantaneously.

That receptor replenishment represents receptor resynthesis is demonstrated by the right-hand graph of Fig. 12. Injection of 50 μl of a 10^{-3} M puromycin solution into one uterine horn from the vagina at

Table 1. Properties of microsomal and cytosol estradiol receptors

Properties	Microsomal receptor	Cytosol receptor
Sed. coefficients	3·5 S 4·5 S (±0·4 M KCL)	4 S(4·2*) 5 S(5·5*) (+0·4 M KCL)
M.W. by gelfiltration	47,000 81,000	76,000* 133,000*
Mobility in agar electroph.	"basic"(β_1) "acidic"(α_2)	"acidic" (α_2)
Estrogens bound	E-1=E-2=E-3 E-2>E-3,[X]	E-1=E-2=E-3 E-2>E-3,[X]
Effect of warming	3·5 $S_{(b)}$ ⟶ 4·5 $S_{(b)}$	4 S ⟶ 5 S
Kinetics of transition	2nd order for dimerization	2nd order for dimerization? (0·4 M KCL, pH 7·5)
Effect of pH 6·5 ⟷ 7·5	3·5 S_b $\underset{+H^{\oplus}}{\overset{-H^{\oplus}}{\rightleftharpoons}}$ 4·5 S_b; 3·5 S_a 4·5 S_a	4 S $\underset{+H^{\oplus}}{\overset{-H^{\oplus}}{\rightleftharpoons}}$ 5 S
Effect of 2M urea (0·4 M KCL, pH 7·5)	3·5 S ⟵ (4·5 S) unstable	4 S $\xrightarrow{heat}$ 5 S stable

* From Notides and Nielsen[9].

5 h after estradiol was injected i.p. slowed the receptor replenishment in the treated horn down until the puromycin was used up. The left-hand graph shows a drop in receptor concentration below the initial level when puromycin was given 30 min prior to estradiol. Since the antibiotic does not interfere with the binding of estradiol to the receptor, receptor synthesis in the absence of estradiol must be assumed.

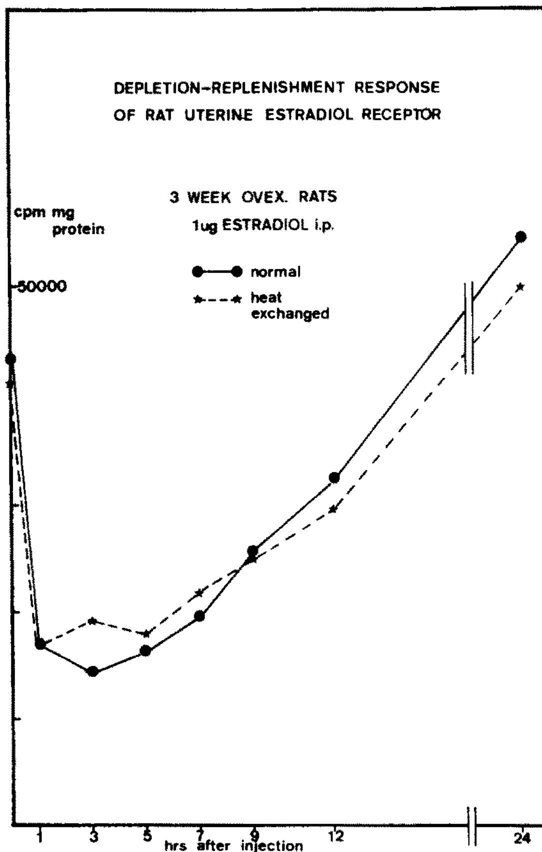

Fig. 11. Depletion-replenishment response of rat uterine estradiol cytosol receptor.

Receptor synthesis and degradation indeed go on in the uteri of ovariectomized and of ovariectomized + hypophysectomized rats, as we saw in a long-term experiment, the results of which are shown in Fig. 13. The animals were kept at 22°C and a 12 h light–dark period. Groups of 3 animals were killed between 8 and 10 a.m. We do not know what governs the fluctuating receptor pattern. It is not due to inter-assay or individual variations. They are too small to show on this figure.

The last two observations might appear to digress from the point at issue, but we have reason to suspect that the extent and the type of response in a depletion–replenishment experiment depend on the starting levels of receptor concentration. It is therefore necessary to keep all conditions as constant as possible, particularly the priming sequence and the pre-experimental pause.

Experiments to gain insight into the physiological role of the microsomal receptors, especially the basic variety, were done with ovariectomized and estradiol-primed pigs. Groups of 3–5 animals received 4 μg/kg E-2 i.p. at various times before slaughtering. The uteri were removed as quickly as possible and kept on ice until processing. Microsomal extracts were analysed by agargelelectrophoresis, low-ionic-strength cytosols by agargelelectrophoresis and density gradient centrifugation, the combined results being shown in Fig. 14. The concentration/time patterns identify the basic microsomal form as an early product of receptor synthesis followed in sequence by the acidic microsomal receptor, which seems to be an intermediate to the cytosol receptors. Although these were only analysed at low ionic strength, it is noteworthy that the pattern of the unresolved 4–5 S forms allows the interpretation that they derive either from the acidic microsomal receptor or from the 9 S cytosol receptor.

We recently adapted for the pig experiments the intrauterine injection design pursued with rats by dis-

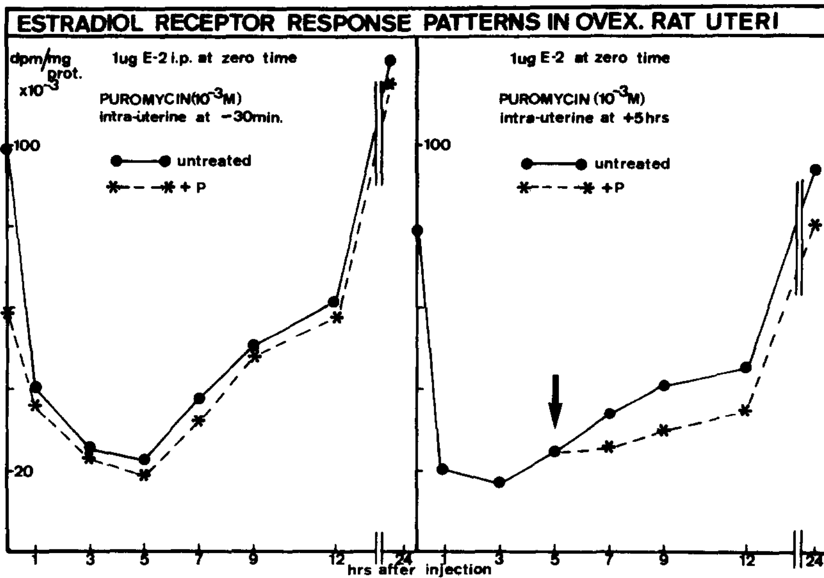

Fig. 12. Effect of puromycin on depletion-replenishment response of uterine estradiol cytosol receptor.

connecting one horn from the corpus uteri. Our first results with this technique not only reaffirm the precursor nature of the basic microsomal receptor, but also show within-minute changes of receptor patterns after intrauterine injection of estradiol and lend further proof to the rapid removal of estradiol-tagged receptor from the cytoplasm.

Considering all the evidence available from *in vitro* and *in vivo* experiments, a chain of events as outlined in Fig. 15 could come very close to the *in vivo* situation. The first product of receptor biosynthesis is the "basic" microsomal 3·5 S receptor. To this, while still structure-bound, a small acidic group is attached to yield the "acidic" 3·5 S receptor. Immediately before or right after release from the endoplasmatic reticulum, the addition of a neutral entity leads to the formation of the cytosol 4 S receptor. At this point the pathway branches. In the absence of hormone, the 4 S receptor is compounded in some unknown fashion to the 9 S form, from which it can be recalled. In the presence of estradiol, the 4 S receptor dimerizes to the 5 S receptor which then immediately leaves the soluble phase of the cytoplasm and enters the nucleus for action and subsequent destruction.

We are aware of the fact that it is provocative to interpret the cytosol 5 S receptor as a dimer of the 4 S molecule. At present, the balance of experimental evidence apparently speaks against this assumption, although supporting evidence emerges

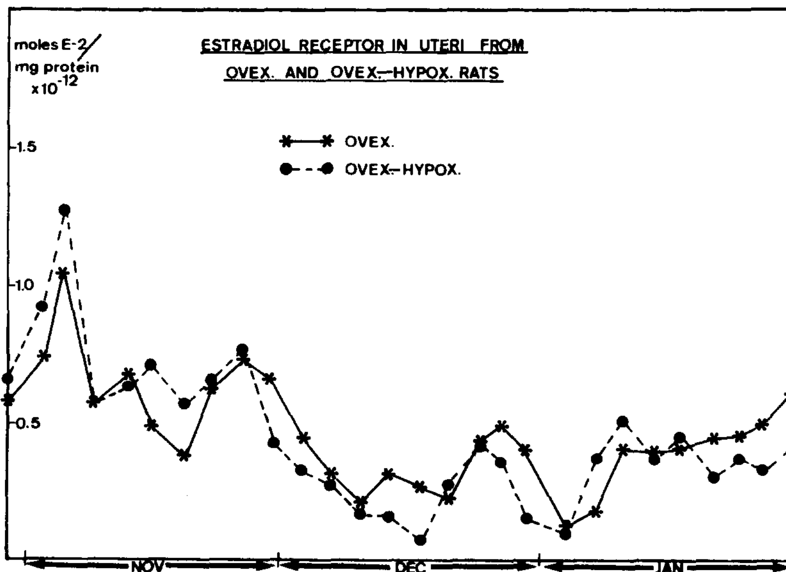

Fig. 13. Long-term patterns of uterine estradiol cytosol receptor concentrations in ovariectomized and ovariectomized + hypophysectomized rats.

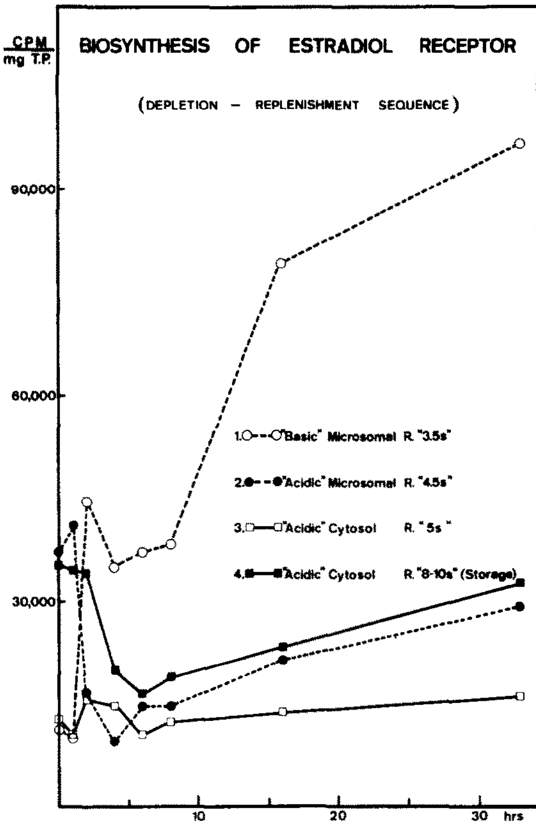

Fig. 14. Depletion-replenishment response of pig uterus estradiol cytoplasmic receptors. Specific activity of [6,7^{3}H]-estradiol: 34 Ci/m Mol; purity 96·5%. TP = tissue protein calculated from: total protein (Lowry)-serum albumin (immunoassay) × 1·67.

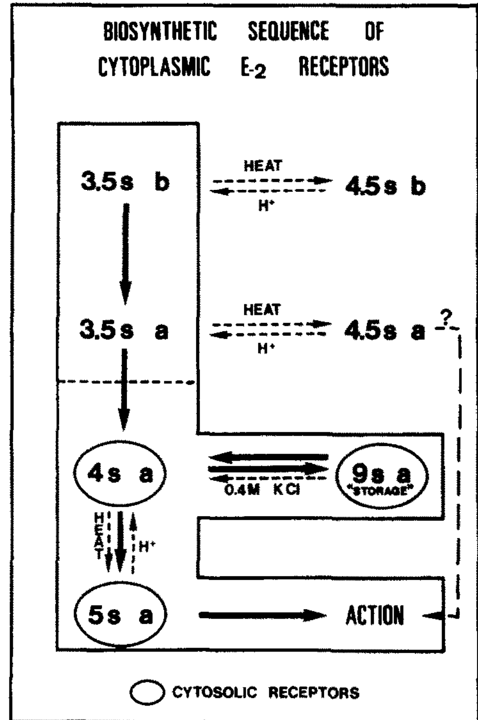

Fig. 15. Model of interrelationship of cytoplasmic estradiol receptors.

from the data on microsomal receptors. Since microsomal receptor dimers—certainly the basic one and most probably also the acidic dimer—can only be produced *in vitro* and since the factors governing aggregation and segregation of both the microsomal and the cytosol receptors are virtually identical, we believe the assumption to be quite reasonable and hope to prove it experimentally beyond doubt. For steric reasons such an active receptor dimer would have to have an upside-down or head-to-tail structure. At this point we prefer not to speculate on its mode of action, but rather conclude by speculating that the other steroid hormones interact with their cytoplasmic receptors in the same way as estradiol, since last year, we described the presence of receptors for dihydrotestosterone and progesterone in the microsomal fraction of pig uteri [7].

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